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(54) Title: INHIBIN AND METHOD OF PURIFYING SAME

(57) Abstract

Two 32,000-dalton proteins with inhibin activity were isolated from porcine follicular fluid using heparin-Sepharose affinity chromatography, followed by gel filtration on Sephacryl S-200 and then four steps of reverse-phase high-performance liquid chromatography. Each isolated molecule is composed of two chains having molecular weights of about 18,000 and about 14,000 daltons, respectively, which are bound together by disulfide bonding. Microsequencing revealed the NH_2-terminal portion of the 18K chain of both to be Ser-Thr-Ala-Pro-Leu-Pro-Trp-Pro-Trp-Ser-Pro-Ala-Ala-Leu-Arg-Leu-Leu-Gln-Arg-Pro-Pro-Glu-Glu-Pro-Ala-Val, of one of the 14K chains to be Gly-Leu-Glu-Cys-Asp-Gly-Arg-Thr-Asn-Leu-Cys-Cys-Arg-Gln-Gln-Phe-Phe-Ile-Asp-Phe-Arg-Leu-Ile-Gly-Trp, and of the other 14K chain to be Gly-Leu-Glu-Cys-Asp-Gly-Lys-Val-Asn-Ile-Cys-Cys-Lys-Lys-Gln-Phe-Phe-Val-Ser-Phe-Lys-Asp-Ile-Gly-Trp-Asn-Asp-Trp-Ile-Ile-Ala-Pro. Both proteins have now been completely characterized, each having a first chain 134 residues long linked by disulfide bonding to a second chain 116 or 115 residues long. The first chain is believed to be glycosylated, which accounts for the disparity between the number of residues and the apparent molecular weight of 18K. These 32K proteins specifically inhibit basal secretion of FSH, but not of LH, in a rat anterior pituitary monolayer culture system. The half-maximal effective dose of one is 450 pg/ml and of the other is 900 pg/ml.

INHIBIN AND METHOD OF PURIFYING SAME

The present invention relates to a protein having inhibin activity isolated substantially to homogeneity from bodily material obtained from porcine animals. The invention also relates to a method of purifying porcine inhibin.

BACKGROUND OF THE INVENTION

The concept of inhibin as a water-soluble substance produced in the gonad but acting specifically at the pituitary level to inhibit the secretion of follicle-stimulating hormone (FSH) was postulated by McCullagh in 1932 (Science, 76, 19-20). Such preferential regulation of the gonadotropin secretion has generated a great deal of interest and has prompted many laboratories in the past fifty years to attempt to isolate and characterize this substance from extracts of testis, spermatozoa, rete testis fluid, seminal plasma and ovarian follicular fluid, using various bioassays. Although many reports have appeared in the literature claiming the purification of inhibin-like material with molecular weights ranging from 5,000 to 100,000 daltons, subsequent studies have shown that these substances were either not homogenous or did not have the high specific activity expected of true inhibin (de Jong, Molecular & Cellular Endocrin., 13, 1-10 (1979)). Materials having inhibin activity may be used to regulate fertility in mammalian animals, particularly male animals.

SUMMARY OF THE INVENTION

In accordance with the present invention, two proteins both having a molecular weight of about 32,000 daltons and having inhibin activity have been successfully isolated from porcine follicular fluid. These two proteins have been completely characterized using microsequencing and molecular biological methods.

The proteins were isolated to substantial homogeneity from material obtained from the bodies of porcine animals and are hereinafter referred to as Protein A and Protein B. Each protein has a molecular weight of about 32,000 daltons (32K) and is composed of two polypeptide chains having molecular weights of 18,000 and 14,000 daltons, respectively, the chains being linked together in the biologically active protein by disulfide bonding. Microsequencing showed that the amino-terminal amino acid residue sequence of the 18,000 dalton (18K) chain of both proteins was Ser-Thr-Ala-Pro-Leu-Pro-Trp-Pro-Trp-Ser-Pro-Ala-Ala-Leu-Arg-Leu-Leu-Gln-Arg-Pro-Pro-Glu-Glu-Pro-Ala-Val and that the first six residues of both amino-terminals of the 14,000 dalton (14K) chains were the same, namely Gly-Leu-Glu-Cys-Asp-Gly. It was also ascertained that the first ten residues of the 14K chain of Protein B were Gly-Leu-Glu-Cys-Asp-Gly-Arg-Thr-Asn-Leu. Proteins A and B have now been completely characterized as a result of further microsequencing and the use of molecular biological techniques. Each 32K protein exhibits inhibin activity in that it specifically inhibits basal secretion of FSH but does not inhibit basal secretion of luteinizing hormone (LH).
The individual chains are not biologically active.

Purification of porcine inhibin to substantial homogeneity, i.e., about 90% by weight of total protein in the fraction, was achieved through a combination of protein separation procedures including heparin-Sepharose affinity chromatography, gel filtration and reverse-phase, high-performance liquid chromatography (RP-HPLC).

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows chromatograms representing initial steps of purifications of inhibin proteins from porcine follicular fluid (PFF) under conditions described as follows:

(a) Heparin-Sepharose affinity chromatography of PFF. The inhibin protein was eluted with 1 M NaCl in 0.01 M Tris-HCl, pH 7.

(b) Sephacryl S-200 gel filtration of the PFF inhibin proteins. The effluent fractions indicated by the solid bar near the bottom of Figure 1a were pooled, dialyzed and then divided into eight equal portions for this gel filtration. The profile from one of the columns is indicative of each column and is shown.

(c) RP-HPLC purification of the inhibin proteins recovered from gel filtration. The components of the active region from the gel filtration, as determined by _in vitro_ bioassay and designated by the solid bar in Figure 1b, were pooled, lyophilized and, after dissolving in 0.2 N acetic acid, applied directly onto a Vydac C4 column and eluted with the indicated gradient of acetonitrile in the TEAP buffer system at 9 ml/3 min. Two inhibin proteins, Proteins A and B, indicated by the solid bars in Figure 1c, were recovered.

FIGURE 2 shows chromatograms of RP-HPLC purification of inhibin Protein B as follows:

(a) The active fractions designated by the solid bar under B in Fig. 1c were pooled and, after being diluted to 3 times their original volume, applied directly onto a Vydac C4 column and eluted with the indicated gradient of acetonitrile in trifluoroacetic acid (TFA) buffer system at 9 ml/3 min.

(b) The active material denoted by the solid bar in Figure 2a was pooled, diluted to 3 times its original volume and likewise purified on a Vydac Phenyl column with the indicated gradient of acetonitrile in triethylammonium phosphate (TEAP) buffer system at 2 ml/2 min.

(c) The active material accumulated from a number of columns identical to that represented by the chromatogram of Figure 2b was pooled and concentrated on an Aquapore RP-300 column with the indicated gradient of acetonitrile in the TFA buffer system at 0.5 ml/min.

FIGURE 3 shows actual electrophoretic results of sodium dodecylsulfate-polyacrylamide gel

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electrophoresis (SDS-PAGE) analysis of the purified
inhibin Protein B as follows:

    (a) Analysis of Protein B under non-reducing
condition.

    (b) Analysis of Protein B under reducing
condition. The molecular weight standards are indicated
on the left.

    FIGURE 4 shows dose-response curves of the
purified inhibin Protein B (O), as well as the crude PFF
standard (Δ) on the basal secretion of FSH and LH from
rat anterior pituitary cell cultures. The crude PFF
standard was a charcoal-stripped and 40% saturated
ammonium sulfate precipitate of PFF, Schwartz, N. et al.
Proc. Natl. Acad. Sci. USA 74 5721-5724 (1977).

    FIGURE 5 shows chromatograms of RP-HPLC
purification of inhibin Protein A as follows:

    (a) The active fractions designated by the
solid bar under A in Fig. 1c were pooled and, after
being diluted to 3 times their original volume, applied
directly onto a Vydac C4 column and eluted with the
indicated gradient of acetonitrile in trifluoroacetic
acid (TFA) buffer system at 9 ml/3 min.

    (b) The active material denoted by the solid
bar in Figure 5a was pooled, diluted to 3 times its
original volume and likewise purified on a Vydac Phenyl
column with the indicated gradient of acetonitrile in
triethylammonium phosphate (TEAP) buffer system at 2
ml/2 min.

    (c) The active material accumulated from a
number of columns identical to that represented by the
chromatogram of Figure 5b was pooled and concentrated on
an Aquapore RP-300 column with the indicated gradient of
acetonitrile in the TFA buffer system at 0.5 ml/min.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

    Using a multi-step procedure, two 32,000 dalton
peptides were isolated to substantial homogeneity from
porcine follicular fluid (PFF). Each protein (Protein A

and Protein B) is composed of two chains of 18K and 14K, respectively, and the chains of the intact molecule are held together by disulfide bonding, the linkage between the chains being necessary for biological activity. An amino acid analysis of the total protein has been performed for each, and the amino acid residue sequence of the amino-terminus of each chain has been determined by microsequencing. The amino-terminal sequence of the 18K chain of each protein was found to be: Ser-Thr-Ala-Pro-Leu-Pro-Trp-Pro-Trp-Ser-Pro-Ala-Ala-Leu-Arg-Leu-Leu-Gln-Arg-Pro-Pro-Glu-Glu-Pro-Ala-Val. As a result of further work, it is believed that both 18K chains have exactly the same sequence of 134 amino acid residues and have a free acid C-terminus.

Microsequencing initially showed that the amino-terminal sequences of the 14K chains of the first six residues of both chains were the same and that the first ten residues of the 14K chain of Protein B were Gly-Leu-Glu-Cys-Asp-Gly-Arg-Thr-Asn-Leu. Further sequencing showed that the next 15 residues were X_{11}-X_{12}-Arg-Gln-Gln-Phe-Phe-Ile-Asp-Phe-Arg-Leu-X_{23}-Gly-Trp, with X_{11} possibly being Ser, with X_{12} likely being Cys and with X_{23} being Ile or Leu. Later it was determined that both X_{11} and X_{12} were Cys and that X_{23} was Ile. The ability to sequence the chains demonstrates that the protein has been purified to at least about 90% by weight of total protein as do the sharp elution peaks of the protein in the final chromatographic purification step.

The C-termini of all three chains are free acid. Further microsequencing showed that the N-terminus of the 14K chain of Protein A has the following sequence: Gly-Leu-Glu-Cys-Asp-Gly-Lys-Val-Asn-Ile-Cys-Cys-Lys-Lys-Gln-Phe-Phe-Val-Ser-Phe-Lys-Asp-Ile-Gly-Trp-Asn-Asp-Trp-Ile-Ile-Ala-Pro.

Protein A has now been completely characterized and found to include a 134-residue chain (the 18K chain)

that is linked by one or more disulfide bridges to a
116-residue chain (the 14K chain) which appears to
include at least one internal disulfide bridge.
Protein B has the same 18K chain linked to a homologous
14K chain.

Each 32K protein is acidic, having a pKa of
4.8, and is generally soluble in aqueous media. Each is
glycosylated to a limited extent, as determined by
limited affinity to concanavalin A. Each 32K protein
exhibits inhibin activity in that it specifically
inhibits basal secretion of FSH in a rat anterior
pituitary monolayer culture system. Protein B exhibits
a half-maximal effective dose of 450 pg/ml, as
calculated by the computer program BIOPROG, and Protein
A exhibits an ED_{50} of 900 pg/ml. Each 32K protein is
useful for regulating fertility of mammalian animals,
particularly males.

Each 32K protein has apparent similarities to a
56,000 dalton (56K) protein isolated from bovine
follicular fluid, a protein composed of two chains of
molecular weights 44,000 and 14,000 daltons (44K and
14K) bound together by disulfide bonds. The first three
amino-terminal residues of the 44K bovine chain reported
by Robertson et al., Bio. Chem. Biophys. Res. Commun.
176 220-226 (1985) are different from either of the two
chains of either of the 32K porcine proteins that were
isolated, but the second and third residues of the 14K
chain of the 56K bovine peptide are identical to those
of the 14K chain of both of the 32K porcine peptides.
The full relationship, if any, between the 32K porcine
proteins and the 56K bovine protein is not known at this
time.

Each 32K protein is definitely different from
the "alpha-inhibins" Ramasharma, K. et al. Science 223,
1199-1201 (1984); Li, C. H. et al., Proc. Natl. Acad.
Sci. USA 82 4041-4044 (1985) and "beta-inhibin" Seidah,
N. FEBS Letter 175 349-354 (1985) recently isolated and

characterized from human seminal plasma. "Alpha-inhibins" are reported to be single-chain polypeptides all derived from a 92-amino-acid-residue parent molecule. However, neither a synthetic replicate of the 31-amino-acid NH_2-terminal fragment of alpha-inhibin-52 nor the native product inhibited the basal FSH-releasing activity in the rat anterior pituitary cell culture, Yamashiro, D. et al., <u>Proc. Natl. Acad. Sci.</u> USA <u>81</u> 5349-5402 (1984). "Beta-Inhibin" is described as a single-chain polypeptide with 94 amino acid residues, with the bioactive core allegedly residing at the COOH-terminal 67-94 fragment, Arbatti,N. et al. <u>FEBS Letters, 181,</u> 57-63 (1985). This fragment has been synthesized, but it is found to be inactive, up to a concentration of 5 µg/ml, to inhibit the basal secretion of FSH in the rat anterior pituitary cell culture assay. The bioassay methods (for "inhibin activity") used in these two laboratories, Ramasharma et al. <u>supra.</u> and Li et al. <u>supra.</u>, are substantially different from those described herein.

In an inhibin purification procedure according to the invention, porcine inhibin is isolated from crude extract material obtained from a porcine animal body, specifically porcine follicular fluid (PFF) although other appropriate bodily extracts might be used, by successive purification procedures that include heparin-Sepharose affinity chromatography, gel filtration and at least one and preferably several RP-HPLCs of different conditions of stationary phase and/or mobile phase. The same procedure is useful in obtaining a desired mammalian inhibin protein from a crude extract resulting from recombinant DNA processes.

In the preferred procedure by which porcine inhibin was first isolated to substantial purity, PFF was first purified by heparin-Sepharose affinity chromatography, next by gel filtration on Sephacryl S-200 gel and then with four successive RP-HPLCs using

different mobile phase gradients and/or derivatized silica supports. Preferably stationary phases having relatively low hydrophobicity are used, with C3-C8 columns being preferred and C3-C5 and phenyl columns being particularly preferred. Solute specificity of the mobile phase is preferably adjusted by varying the concentration of an organic component, particularly acetonitrile. Although a single RP-HPLC fractionation significantly increases the purity relative to the gel-filtrated material, two or more, and preferably four, RP-HPLC purifications are generally performed subsequent to sucessive treatment by heparin-Sepharose chromatography and gel filtration.

The starting material for the procedure was frozen PFF that was procured from J.R. Scientific, Woodland, CA. Approximately 18 liters of such frozen PFF were processed in 250 ml batches to isolate the inhibin product. The first step of the purification was heparin-Sepharose affinity chromatography, in which the protein is adsorbed to the Sepharose-bound heparin moieties under application conditions, and the adsorbed inhibin material is recovered by 1M NaCl elution. This step greatly expedites the purification procedure for crude extracts because it allows a relatively large volume of a crude extract, such as PFF, to be processed fairly rapidly while recovering an amount of protein exhibiting total inhibin activity equal to at least 90% of that of the curde extract.

Throughout the purification procedure, the inhibin bioactivity was monitored by an _in vitro_ bioassay using rat anterior pituitary monolayer culture, Vale, W. et al. _Endocrinology_, _91_, 562-572 (1972). In brief, 21-day-old female rat anterior pituitaries are collected, enzymatically dispersed and plated in 10% fetal bovine serum in HDMEM (GIBCO Laboratories, Santa Clara, CA.) into 24-well tissue culture plates (Falcon Plastic, Oxnard, CA.) on day 1. On day 2, the medium is

changed to 1% fetal bovine serum in HDMEM, and the
sample is added. Incubation is continued for another 48
hours. The medium is then harvested, and the LH and FSH
contents are determined by radio-immunoassay (RIA) using
materials provided by The Pituitary Hormone Program of
NIADDKD. In this assay, the inhibin proteins inhibit
the basal release of FSH only but not that of LH, as
compared to control cells that receive the incubation
medium only.

For the detection of inhibin activity in the
various column fractions, aliquots ranging from 0.01% to
0.1% by volume were removed, and after adding 100 µg
human serum albumin in 100 µl water, the solvents were
evaporated in a Speed-Vac concentrator (Savant,
Hicksville, N.Y.). The residue was redissolved in 3 ml
1% fetal bovine serum in HDMEM, filtered through a
Millex-GS 0.22 um filter (Millipore Corp., Bedford, MA)
and assayed in duplicate. To speed up the bioassays
during the purification process, only basal inhibition
of FSH secretion exerted by the inhibin activity was
determined and plotted in the region where the inhibin
proteins were expected to migrate in the chromatograms.

To perform the heparin-Sepharose affinity
chromatography, a 500 ml bottle of frozen PFF was
defrosted, and the cell debris were spun down in a
Beckman J2-21 centrifuge (Beckman Instruments, Inc.,
Palo Alto, CA.) using a JA-20 rotor at 10,000 rpm for 30
minutes. One half of the supernatant (250 ml) was
diluted to 10 times its volume by the addition of 2,250
ml of 0.01 M Tris-HCl containing 0.1 M NaCl, pH 7, in a
4 liter Erlenmeyer flask and pumped simultaneously via
eight silastic tubes (0.76mm ID) into eight
heparin-Sepharose (Pharmacia Fine Chemicals, Piscataway,
N. J.) columns (3.5 x 9 cm) by two Rabbit 4-channel
peristaltic pumps (Rainin Instrument Co., Inc.,
Emeryville, CA.) at 40 ml/hr per column. After all the
fluid had been pumped through the heparin-Sepharose, the

eight columns were washed simultaneously with 3.5 liters of 0.01 M Tris-HCl, pH 7, containing 0.1 M NaCl in the same manner. The adsorbed proteins with inhibin activity were removed by washing the eight columns

5 simultaneously with 1.3 liters of 0.01 M Tris-HCl containing 1 M NaCl, pH 7, as above, and the wash was collected into fractions of 16 ml. Figure 1a shows a typical elution profile of the inhibin activity material from PFF. The inhibin activity was monitored by the in

10 vitro bioassay described above. The columns were regenerated by further washing with 1.6 liters 2M NaCl in 0.01 M Tris-HCl, pH 7, and re-equilibrated with 3.5 liters 0.01 M Tris-HCl containing 0.1 M NaCl for purification of the remaining 250 ml of PFF.

15   Next, the material was fractionated by gel filtration to separate proteins generally according to their molecular weights. The fractions having inhibin activity extracted by the eight heparin-Sepharose columns were pooled (400 ml) and dialyzed overnight to

20 remove NaCl in a 28.6 mm cylinder diameter Spectrapor No. 3 membrane tubing with M_r cutoff at 3,500 (Spectrum Medical Industries, Inc., Los Angeles, CA.) against 16 liters of 30% acetic acid. The retained fluid was centrifuged, as above, to remove a white

25 precipitate, and the supernatant was divided into eight equal portions for applying to eight 5 x 100 cm Sephacryl S-200 superfine columns (Pharmacia Fine Chemicals, Piscataway, N. J.). Each column was eluted with 30% acetic acid at 20 ml for 22 min., and the

30 column fractions were monitored by UV absorption at 280 nm and by bioassay.

        Fig. 1b shows the elution profile of the inhibin material purified in a Sephacryl S-200 column. Two elution zones of approximately equal amounts of

35 activity were detected, one eluting at M_r about 30,000 and the other at M_r about 12,000. However, based upon the protein concentration as determined by UV

absorption, the low-molecular-weight zone contains much
higher specific activity. As a result, this region was
selected for further purification by RP-HPLC.

The low-molecular-weight inhibin protein from
the eight S-200 columns was pooled and lyophilized. The
lyophilized material (40 mg) was dissolved in 40 ml 0.2N
acetic acid and filtered through a Millex-HA 0.45 μm
filter (Millipore Corp., Bedford, MA.). The filtrate
was applied directly onto a 1 x 25 cm Vydac 5-μm
particle-size C4 column (The Separations Group,
Hesperia, CA.) and developed with a gradient of TEAP
buffer as shown in Figure 1c. In the TEAP system,
buffer A consists of 0.25 N. triethylammonium phosphate,
pH 3, and buffer B is 80% acetonitrile in buffer A.
After all the filtrate had been loaded, the column was
washed with the aqueous buffer A until the UV absorption
reached baseline. The fractions exhibiting inhibin
activity were separated in a Beckman 332 gradient liquid
chromatography system (Beckman Instruments, Inc.,
Berkely, CA.) equipped with a Spectroflow 757 UV
detector (Kratos Analytical Instruments, Ramsey, N. J.),
a Soltec 220 recorder (Soltec Corp., Sun Valley, CA.)
and a Redirac 2112 fraction collector (LKB Instruments,
Inc., Gathersburg, MD.). Two zones of substantial
inhibin activity were detected, with more activity
exhibited by the earlier eluting zone (inhibin Protein
A) than the later eluting zone (inhibin Protein B), see
Figure 1c.

Inhibin Protein B from the various columns were
pooled and further purified by two more RP-HPLC steps.
The first step uses a 1 x 25 cm Vydac 5-μm-particle-size
C4 column and a trifluoroacetic acid (TFA) buffer system
(Figs. 2a) and the second step employs a 1 x 25 cm Vydac
5-μm-particle-size Phenyl column and the TEAP buffer
system, as shown in Fig. 2b. In the TFA system, buffer
A contains 1 ml trifluoracetic acid in 999 ml water and
buffer B is 1 ml trifluoroacetic acid in 199 ml water

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and 800 ml acetonitrile. Finally, inhibin Protein B
accumulated from a few batches was concentrated by
RP-HPLC using a 0.46 x 25 cm Aquapore RP-300 10
um-particle-size column (Brownlee Labs., Santa Clara,
5   CA.) and the TFA buffer system as shown in Fig. 2c.
Altogether, a total of approximately 60 µg of inhibin
Protein B was purified from the 18 liters of PFF.

Inhibin Protein A from the various columns was
pooled and further purified by two more RP-HPLC steps.
10   The first step uses a 1 x 25 cm Vydac 5-µm-particle-size
C4 column and a·trifluoroacetic acid (TFA) buffer system
(Figs. 5a) and the second step employs a 1 x 25 cm Vydac
5-µm-particle-size Phenyl column and the TEAP buffer
system, as shown in Fig. 5b. In the TFA system, buffer
15   A contains 1 ml trifluoracetic acid in 999 ml water and
buffer B is 1 ml trifluoroacetic acid in 199 ml water
and 800 ml acetonitrile. Finally, inhibin Protein A
accumulated from a few batches were concentrated by
RP-HPLC using a 0.46 x 25 cm Aquapore RP-300 10
20   µm-particle-size column (Brownlee Labs., Santa Clara,
CA.) and the TFA buffer system as shown in Fig. 5c.
Altogether, a total of approximately 600 µg of inhibin
Protein A was purified from the 18 liters of PFF.

Amino acid analyses of the substantially
25   homogeneous inhibin Proteins A and B were performed as
described in Bohlen P., et al. Anal. Biochem. 126
144-156 (1982), and the results are shown in Table 1
below.

TABLE 1

AMINO ACID COMPOSITION OF PURIFIED INHIBIN
PROTEIN FROM PORCINE FOLLICULAR FLUID

	Amino Acid	Protein A*	Protein B*
5	Asx	18.5 ± 1.6	21.0 ± 1.1
	Thr	11.5 ± 0.5	13.5 ± 0.6
	Ser	16.3 ± 1.4	18.1 ± 0.7
	Glx	21.3 ± 1.4	22.5 ± 0.5
	Gly	19.9 ± 1.9	22.4 ± 0.7
10	Ala	19.7 ± 1.4	22.0 ± 0.6
	Val	14.1 ± 1.8	14.4 ± 0.4
	Met	6.1 ± 0.4	5.8 ± 0.3
	Ile	14.6 ± 1.7	10.3 ± 0.2
	Leu	27.3 ± 2.1	27.8 ± 0.8
15	Tyr	10.7 ± 1.3	11.4 ± 0.4
	Phe	11.6 ± 1.4	10.0 ± 0.2
	His	14.1 ± 1.2	7.9 ± 0.6
	Trp	4.4 ± 0.3	3.9 ± 0.4
	Lys	11.9 ± 0.9	5.4 ± 0.2
20	Arg	13.9 ± 0.6	15.8 ± 0.4
	Cys**	14.4 ± 0.2	14.2 ± 0.9
	Pro	29.2 ± 0.9	29.6 ± 1.1

* Data corresponds to the mean $\pm$ SD of four analyses
and normalized to a protein of 32,000 daltons.

25 ** Cysteine was determined as cysteic acid after
performic acid oxidation.

The inhibin Protein B from the final RP-HPLC
purification was analyzed under reducing and
non-reducing conditions in 1-mm-thick 15% acrylamide gel
30 according to the method of Laemmli, V., Nature 227
680-685 (1970). The protein was revealed by silver
staining reagent (BIO-RAD, Richmond CA.). The following
molecular weight standards were used to calibrate the
gel: bovine serum albumin (M_r =67,000), ovalbumin
35 (M_r=43,000), alpha-chymotrypsinogen (M_r= 25,700) and

lysozyme (M_r = 14,500). In non-reducing condition, 2
µg of inhibin protein in 20 µl water was incubated with
20 µl sample buffer (0.152 M Tris-HCl, pH 6.8,
containing 20% glycerol (V/V), 4% sodium dodecylsulfate
and 0.04% bromphenol blue) for 1 hour at 37°C prior to
loading onto the gel. The electrophoresis was performed
at a constant 200 volts for 6 hours at room
temperature. In reducing condition, 2 ug of protein was
incubated, first with 20 µl of 0.02 M dithiothreitol for
15 min at 37°C and then 20 µl of sample buffer was added
and the incubation continued for one more hour before
the sample was applied to the gel. Electrophoresis was
carried out as above, except that 0.005 M dithiothreitol
was included in the electrophoretic buffer. On SDS-PAGE
under non-reducing condition, inhibin Protein B showed a
single band migrating at M_r 32,000 (See Figure 3a).
Under reducing condition, inhibin Protein B separated
into two bands, one migrating at M_r 18,000 and the
other M_r 14,000 (See Figure 3b). Similar
electrophoresis of Protein A showed two such bands plus
one at M_r 8,000 which was felt to possibly indicate
the presence of impurity.

NH_2-terminal sequence analyses of the 18K and
14K chains of 32K inhibin Proteins A and B were
accomplished by first separating the two chains by
SDS-PAGE under reducing conditions, followed by
electro-blotting of the separated protein chains onto
GF/C paper which was subsequently punched out for direct
microsequencing. Briefly, each inhibin protein (12-15
µg) was electrophoresed on 0.5-mm-thick 15% acrylamide
gel (8 x 10 cm) under reducing condition, as described
above. After electrophoresis, the gel was immediately
washed 3 times with 0.5% NP-40 (Sigma Chemical Co., St.
Louis, MO.) in 0.5% acetic acid at room temperature.
The gel was then placed between 2 sheets of
trifluoracetic acid-activated GF/C paper (Whatman,
Clifton, N.J.) which had been wet briefly with the

blotting buffer (2% acetic acid containing 0.5% NP-40).
One more activated GF/C paper was placed on the cathode
side in case the protein was not completely absorbed by
the first paper. The outer surface of the GF/C paper
5   was protected by Whatman No. 3 filter paper, and the
whole assembly inserted between two Scotch-Brite pads
held together by a plastic grid (BIO-RAD, Richmond,
CA). This was electrophoresed in the blotting buffer at
a constant voltage of 60 V for 16 hours at 4°C to
10   transfer the positively charged proteins onto the GF/C
paper. The electrolytically transferred protein chains
were revealed with Coomassie blue staining. 1.2 cm
diameter circles were punched out of the stained protein
bands and subjected directly to microsequencing as
15   described above.

Microsequencing, as described in Esch, F. Anal.
Biochem. 136, 39-47, 1984, of intact inhibin protein
beginning at the NH_2-terminus had consistently
revealed two residues of approximately equal
20   concentration at every cycle, indicating each protein is
composed of two chains. Based upon the results from
multiple sequencing analyses of both intact and reduced
inhibin proteins, the NH_2-terminal residues of the 18K
chain of each inhibin protein are Ser-Thr-Ala-Pro-Leu-
25   Pro-Trp-Pro-Trp-Ser-Pro-Ala-Ala-Leu-Arg-Leu-Leu-Gln-
Arg-Pro-Pro-Glu-Glu-Pro-Ala-Val. The NH_2-terminal
residues of the 14K chain of inhibin Protein B are
Gly-Leu-Glu-Cys-Asp-Gly-Arg-Thr-Asn-Leu-Cys-Cys-Arg-
Gln-Gln-Phe-Phe-Ile-Asp-Phe-Arg-Leu-Ile-Gly-Trp-, and
30   the NH_2-terminal residues of the 14K chain of inhibin
Protein A are Gly-Leu-Glu-Cys-Asp-Gly-Lys-Val-Asn-Ile-
Cys-Cys-Lys-Lys-Gln-Phe-Phe-Val-Ser-Phe-Lys-Asp-Ile-
Gly-Trp-Asn-Asp-Trp-Ile-Ile-Ala-Pro-.

Once a substantial portion of the sequence of
35   the three chains of inhibin protein are known, the mRNA
encoding the chains can be isolated, and the cDNA's can
be synthesized by recombinant DNA techniques. Messenger

RNA (mRNA) is obtained from ovarian follicules which produce inhibin, and then cDNA is synthesized from the mRNA by reverse transcription. The cDNA is inserted into a cloning vector which is used to transform a

5   suitable host to create a cDNA library.

Based upon the known partial amino acid residue sequences of the three inhibin chains, labelled oligonucleotides are synthesized for detecting cDNA corresponding to each chain. Because of the degeneracy

10   of the genetic code, mixed hybridization probes are prepared and used as probes. These probes are then used to select, from the library, cDNA clones that contain gene sequences encoding the chains. cDNA libraries may also be screened by immunological expression assay with

15   antibody raised against inhibin or one of the inhibin chains. Immunological expression assay may also be used to confirm screening with hybridization probes.

From selected clones, cDNA is excised and inserted into appropriate vectors under the control of

20   promotor sequences, and the vectors are transformed into cell lines for expression of the recombinant inhibin chains. Although vectors containing the genes for an appropriate pair of chains could conceivably be transformed into the same cell line, for simplicity,

25   vectors for expression of each chain are preferably transformed separately into cell lines. The individual inhibin chains can then be isolated from the cellular material and/or the cell culture medium and are useful as intermediates for the preparation of the bioactive

30   inhibin. Appropriate quantities of the 18K and the 14K chains are then subjected to oxidizing conditions which promote disulfide bonding between the chains to produce inhibin.

The foregoing molecular biology techniques may

35   also be used to read the gene sequences encoding the separate inhibin chains, and thereby completely characterize the protein chains. Protein A has now been

completely characterized and found to include a
134-residue chain (the 18K chain) that is linked by one
or more disulfide bridges to a 116-residue chain (the
14K chain). The 134-residue chain has the following
formula: H-Ser-Thr-Ala-Pro-Leu-Pro-Trp-Pro-Trp-Ser-Pro-
Ala-Ala-Leu-Arg-Leu-Leu-Gln-Arg-Pro-Pro-Glu-Glu-Pro-Ala-
Val-His-Ala-Asp-Cys-His-Arg-Ala-Ser-Leu-Asn-Ile-Ser-Phe-
Gln-Glu-Leu-Gly-Trp-Asp-Arg-Trp-Ile-Val-His-Pro-Pro-Ser-
Phe-Ile-Phe-His-Tyr-Cys-His-Gly-Gly-Cys-Gly-Leu-Pro-Thr-
Leu-Pro-Asn-Leu-Pro-Leu-Ser-Val-Pro-Gly-Ala-Pro-Pro-Thr-
Pro-Val-Gln-Pro-Leu-Leu-Leu-Val-Pro-Gly-Ala-Gln-Pro-Cys-
Cys-Ala-Ala-Leu-Pro-Gly-Thr-Met-Arg-Ser-Leu-Arg-Val-Arg-
Thr-Thr-Ser-Asp-Gly-Gly-Tyr-Ser-Phe-Lys-Tyr-Glu-Thr-Val-
Pro-Asn-Leu-Leu-Thr-Gln-His-Cys-Ala-Cys-Ile-OH. The
116-residue chain has the following formula: H-Gly-Leu-
Glu-Cys-Asp-Gly-Lys-Val-Asn-Ile-Cys-Cys-Lys-Lys-Gln-Phe-
Phe-Val-Ser-Phe-Lys-Asp-Ile-Gly-Trp-Asn-Asp-Trp-Ile-Ile-
Ala-Pro-Ser-Gly-Tyr-His-Ala-Asn-Tyr-Cys-Glu-Gly-Glu-Cys-
Pro-Ser-His-Ile-Ala-Gly-Thr-Ser-Gly-Ser-Ser-Leu-Ser-Phe-
His-Ser-Thr-Val-Ile-Asn-His-Tyr-Arg-Met-Arg-Gly-His-Ser-
Pro-Phe-Ala-Asn-Leu-Lys-Ser-Cys-Cys-Val-Pro-Thr-Lys-Leu-
Arg-Pro-Met-Ser-Met-Leu-Tyr-Tyr-Asp-Asp-Gly-Gln-Asn-Ile-
Ile-Lys-Lys-Asp-Ile-Gln-Asn-Met-Ile-Val-Glu-Glu-Cys-Gly-
Cys-Ser-OH. This characterization is in agreement with
the earlier analyses of the purified protein material
obtained from PFF; the disparity between the number of
residues in the first chain and the measured value of
18K is accounted for by the presence of the
aforementioned glycosylation. It is believed that a
carbohydrate moiety having a molecular weight of about
3000 daltons is attached to the side chain of the Asn
residue in the 36-position of the first chain.

Protein B has also been characterized and the
18K chain is believed to have the same sequence as
Protein A. The 14K chain has been found to have the
following 115-residue formula: H-Gly-Leu-Glu-Cys-Asp-Gly-
Arg-Thr-Asn-Leu-Cys-Cys-Arg-Gln-Gln-Phe-Phe-Ile-Asp-Phe-

Arg-Leu-Ile-Gly-Trp-Ser-Asp-Trp-Ile-Ile-Ala-Pro-Thr-Gly-
Tyr-Tyr-Gly-Asn-Tyr-Cys-Glu-Gly-Ser-Cys-Pro-Ala-Tyr-Leu-
Ala-Gly-Val-Pro-Gly-Ser-Ala-Ser-Ser-Phe-His-Thr-Ala-Val-
Val-Asn-Gln-Tyr-Arg-Met-Arg-Gly-Leu-Asn-Pro-Gly-Thr-Val-
5   Asn-Ser-Cys-Cys-Ile-Pro-Thr-Lys-Leu-Ser-Thr-Met-Ser-Met-
Leu-Tyr-Phe-Asp-Asp-Glu-Tyr-Asn-Ile-Val-Lys-Arg-Asp-Val-
Pro-Asn-Met-Ile-Val-Glu-Glu-Cys-Gly-Cys-Ala-OH. This
characterization is in agreement with the earlier
analyses of the purified protein material obtained from
10   PFF.

       The invention thus provides polypetides or
relatively short proteins between about 115 residues and
about 134 residues in length which are useful as
intermediates to create disulfide-bonded dimers that are
15   biologically active. More specifically, the invention
provides the three polypeptides set forth as follows:
(a) H-Ser-Thr-Ala-Pro-Leu-Pro-Trp-Pro-Trp-Ser-Pro-Ala-
Ala-Leu-Arg-Leu-Leu-Gln-Arg-Pro-Pro-Glu-Glu-Pro-Ala-Val-
His-Ala-Asp-Cys-His-Arg-Ala-Ser-Leu-Asn-Ile-Ser-Phe-Gln-
20   Glu-Leu-Gly-Trp-Asp-Arg-Trp-Ile-Val-His-Pro-Pro-Ser-Phe-
Ile-Phe-His-Tyr-Cys-His-Gly-Gly-Cys-Gly-Leu-Pro-Thr-Leu-
Pro-Asn-Leu-Pro-Leu-Ser-Val-Pro-Gly-Ala-Pro-Pro-Thr-Pro-
Val-Gln-Pro-Leu-Leu-Leu-Val-Pro-Gly-Ala-Gln-Pro-Cys-Cys-
Ala-Ala-Leu-Pro-Gly-Thr-Met-Arg-Ser-Leu-Arg-Val-Arg-Thr-
25   Thr-Ser-Asp-Gly-Gly-Tyr-Ser-Phe-Lys-Tyr-Glu-Thr-Val-Pro-
Asn-Leu-Leu-Thr-Gln-His-Cys-Ala-Cys-Ile-OH; (b) H-Gly-
Leu-Glu-Cys-Asp-Gly-Arg-Thr-Asn-Leu-Cys-Cys-Arg-Gln-Gln-
Phe-Phe-Ile-Asp-Phe-Arg-Leu-Ile-Gly-Trp-Ser-Asp-Trp-Ile-
Ile-Ala-Pro-Thr-Gly-Tyr-Tyr-Gly-Asn-Tyr-Cys-Glu-Gly-Ser-
30   Cys-Pro-Ala-Tyr-Leu-Ala-Gly-Val-Pro-Gly-Ser-Ala-Ser-Ser-
Phe-His-Thr-Ala-Val-Val-Asn-Gln-Tyr-Arg-Met-Arg-Gly-Leu-
Asn-Pro-Gly-Thr-Val-Asn-Ser-Cys-Cys-Ile-Pro-Thr-Lys-Leu-
Ser-Thr-Met-Ser-Met-Leu-Tyr-Phe-Asp-Asp-Glu-Tyr-Asn-Ile-
Val-Lys-Arg-Asp-Val-Pro-Asn-Met-Ile-Val-Glu-Glu-Cys-Gly-
35   Cys-Ala-OH and (c) H-Gly-Leu-Glu-Cys-Asp-Gly-Lys-Val-Asn-

Ile-Cys-Cys-Lys-Lys-Gln-Phe-Phe-Val-Ser-Phe-Lys-Asp-Ile-
Gly-Trp-Asn-Asp-Trp-Ile-Ile-Ala-Pro-Ser-Gly-Tyr-His-Ala-
Asn-Tyr-Cys-Glu-Gly-Glu-Cys-Pro-Ser-His-Ile-Ala-Gly-Thr-
Ser-Gly-Ser-Ser-Leu-Ser-Phe-His-Ser-Thr-Val-Ile-Asn-His-
Tyr-Arg-Met-Arg-Gly-His-Ser-Pro-Phe-Ala-Asn-Leu-Lys-Ser-
Cys-Cys-Val-Pro-Thr-Lys-Leu-Arg-Pro-Met-Ser-Met-Leu-Tyr-
Tyr-Asp-Asp-Gly-Gln-Asn-Ile-Ile-Lys-Lys-Asp-Ile-Gln-Asn-
Met-Ile-Val-Glu-Glu-Cys-Gly-Cys-Ser-OH. For example,
the 134-residue polypeptides may be combined with one of
the other shorter polypeptides to form biologically
active disulphide-bonded heterodimers.

Substantially pure 32K inhibin or the nontoxic
salts thereof, combined with a pharmaceutically
acceptable carrier to form a pharmaceutical composition,
may be administered to mammals, including humans, either
intravenously, subcutaneously, percutaneously,
intramuscularly or orally for control of fertility.
Administration of inhibin induces decreased fertility in
female mammals and decreases spermatogenesis in male
mammals. Administration of a sufficient amount of
inhibin induces infertility in mammals. Inhibin is also
useful for tests to diagnose infertility.

Such peptides are often administered in the
form of pharmaceutically acceptable nontoxic salts, such
as acid addition salts or metal complexes, e.g., with
zinc, iron or the like (which are considered as salts
for purposes of this application). Illustrative of such
acid addition salts are hydrochloride, hydrobromide,
sulphate, phosphate, maleate, acetate, citrate,
benzoate, succinate, malate, ascorbate, tartrate and the
like. If the active ingredient is to be administered in
tablet form, the tablet may contain a binder, such as
tragacanth, corn starch or gelatin; a disintegrating
agent, such as alginic acid; and a lubricant, such as
magnesium stearate. If administration in liquid form is
desired, sweetening and/or flavoring may be used, and
intravenous administration in isotonic saline, phosphate
buffer solutions or the like may be effected.

Inhibin should be administered under the guidance of a physician, and pharmaceutical compositions will usually contain an effective amount of the peptide in conjunction with a conventional, pharmaceutically-acceptable carrier. The dosage will vary depending upon the specific purpose for which the protein is being administered, and dosage levels in the range of about 0.1 to about 1 milligrams per Kg. of body weight may be used when the protein is administered on a regular basis as a male contraceptive.

Although the method of purification of inhibin has been described primarily in terms of isolation from PFF, innibin can be similarly purified from other crude extracts. The term "crude extracts" as used herein refers to other mammalian body material in addition to follicular fluid, as well as to extracts from organisms including laboratory microorganisms, such as prokaryotes (e.g. E. Coli) and eukaryotes (e.g. S. cerevisiae yeast), which have been transformed by means of state of the art methodology to manufacture mammalian inhibin protein.

Although the invention has been described with regard to its preferred embodiments, which constitute the best mode presently known to the inventors, it should be understood that various changes and modifications as would be obvious to one having the ordinary skill in this art may be made without departing from the scope of the invention which is set forth in the claims appended hereto.

Particular features of the invention are emphasized in the claims which follow.

WHAT IS CLAIMED IS:

     1. A 32,000-dalton protein having a purity of at least about 90% by weight of total proteins, said protein having a pKa of about 4.8, said protein being composed of a first polypeptide chain having a molecular weight of about 18,000 and a second polypeptide chain having a molecular weight of about 14,000 daltons, said first chain and said second chain being linked to each other through disulfide bonding in the active protein, said first chain having an amino-terminal sequence beginning with Ser-Thr-Ala-Pro-Leu-Pro-Trp-Pro-Trp-Ser-Pro-Ala-Ala-Leu-Arg-Leu-Leu-Gln-Arg-Pro-Pro-Glu-Glu-Pro-Ala-Val and said second chain having an amino-terminal sequence beginning with Gly-Leu-Glu-Cys-Asp-Gly, said protein specifically inhibiting basal secretion of follicle-stimulating hormone while not inhibiting basal secretion of luteinizing hormone.

     2. A protein according to claim 1 wherein said second chain has an amino terminal sequence beginning with Gly-Leu-Glu-Cys-Asp-Gly-Arg-Thr-Asn-Leu.

     3. A protein according to claim 1 wherein said second chain has an amino terminal sequence beginning with Gly-Leu-Glu-Cys-Asp-Gly-Arg-Thr-Asn-Leu-X-Cys-Arg-Gln-Gln-Phe-Phe-Ile-Asp-Phe-Arg-Leu-Ile-Gly-Trp, wherein X is an unknown amino acid residue.

     4. A protein according to claim 1 wherein said second chain has the formula: H-Gly-Leu-Glu-Cys-Asp-Gly-Lys-Val-Asn-Ile-Cys-Cys-Lys-Lys-Gln-Phe-Phe-Val-Ser-Phe-Lys-Asp-Ile-Gly-Trp-Asn-Asp-Trp-Ile-Ile-Ala-Pro.

5. A protein according to claim 1 wherein said second chain has the formula: H-Gly-Leu-Glu-Cys-Asp-Gly-Lys-Val-Asn-Ile-Cys-Cys-Lys-Lys-Gln-Phe-Phe-Val-Ser-Phe-Lys-Asp-Ile-Gly-Trp-Asn-Asp-Trp-Ile-Ile-Ala-Pro-Ser-Gly-Tyr-His-Ala-Asn-Tyr-Cys-Glu-Gly-Glu-Cys-Pro-Ser-His-Ile-Ala-Gly-Thr-Ser-Gly-Ser-Ser-Leu-Ser-Phe-His-Ser-Thr-Val-Ile-Asn-His-Tyr-Arg-Met-Arg-Gly-His-Ser-Pro-Phe-Ala-Asn-Leu-Lys-Ser-Cys-Cys-Val-Pro-Thr-Lys-Leu-Arg-Pro-Met-Ser-Met-Leu-Tyr-Tyr-Asp-Asp-Gly-Gln-Asn-Ile-Ile-Lys-Lys-Asp-Ile-Gln-Asn-Met-Ile-Val-Glu-Glu-Cys-Gly-Cys-Ser-OH.

6. A protein according to Claim 1 having an amino acid composition of about Asx 21.0, Thr 13.5. Ser 18.1, Glx 22.5, Gly 22.4, Ala 22.0, Val 14.4, Met 5.8, Ile 10.3, Leu 27.8, Tyr 11.4, Phe 10.0, His 7.9, Trp 3.9, Lys 5.4, Arg 15.8, Cys 14.2, and Pro 29.6.

7. A protein according to Claim 1 having an amino acid composition of about Asx 18.5, Thr 11.5. Ser 16.3, Glx 21.3, Gly 19.9, Ala 19.7, Val 14.1, Met 6.1, Ile 14.6, Leu 27.3, Tyr 10.7, Phe 11.6, His 14.1, Trp 4.4, Lys 11.9, Arg 13.9, Cys 14.4, and Pro 29.2.

8. A protein according to Claim 1 wherein said first chain has the formula: H-Ser-Thr-Ala-Pro-Leu-Pro-Trp-Pro-Trp-Ser-Pro-Ala-Ala-Leu-Arg-Leu-Leu-Gln-Arg-Pro-Pro-Glu-Glu-Pro-Ala-Val-His-Ala-Asp-Cys-His-Arg-Ala-Ser-Leu-Asn-Ile-Ser-Phe-Gln-Glu-Leu-Gly-Trp-Asp-Arg-Trp-Ile-Val-His-Pro-Pro-Ser-Phe-Ile-Phe-His-Tyr-Cys-His-Gly-Gly-Cys-Gly-Leu-Pro-Thr-Leu-Pro-Asn-Leu-Pro-Leu-Ser-Val-Pro-Gly-Ala-Pro-Pro-Thr-Pro-Val-Gln-Pro-Leu-Leu-Leu-Val-Pro-Gly-Ala-Gln-Pro-Cys-Cys-Ala-Ala-Leu-Pro-Gly-Thr-Met-Arg-Ser-Leu-Arg-Val-Arg-Thr-Thr-Ser-Asp-Gly-Gly-Tyr-Ser-Phe-Lys-Tyr-Glu-Thr-Val-Pro-Asn-Leu-Leu-Thr-Gln-His-Cys-Ala-Cys-Ile-OH and is glycosylated.

9. A protein according to Claim 5 wherein
said first chain has the formula: H-Ser-Thr-Ala-Pro-Leu-
Pro-Trp-Pro-Trp-Ser-Pro-Ala-Ala-Leu-Arg-Leu-Leu-Gln-Arg-
Pro-Pro-Glu-Glu-Pro-Ala-Val-His-Ala-Asp-Cys-His-Arg-Ala-
5   Ser-Leu-Asn-Ile-Ser-Phe-Gln-Glu-Leu-Gly-Trp-Asp-Arg-Trp-
Ile-Val-His-Pro-Pro-Ser-Phe-Ile-Phe-His-Tyr-Cys-His-Gly-
Gly-Cys-Gly-Leu-Pro-Thr-Leu-Pro-Asn-Leu-Pro-Leu-Ser-Val-
Pro-Gly-Ala-Pro-Pro-Thr-Pro-Val-Gln-Pro-Leu-Leu-Leu-Val-
Pro-Gly-Ala-Gln-Pro-Cys-Cys-Ala-Ala-Leu-Pro-Gly-Thr-Met-
10   Arg-Ser-Leu-Arg-Val-Arg-Thr-Thr-Ser-Asp-Gly-Gly-Tyr-Ser-
Phe-Lys-Tyr-Glu-Thr-Val-Pro-Asn-Leu-Leu-Thr-Gln-His-Cys-
Ala-Cys-Ile-OH.

10. A protein according to claim 1 wherein
said second chain has the formula:
15   H-Gly-Leu-Glu-Cys-Asp-Gly-Arg-Thr-Asn-Leu-Cys-Cys-Arg-Gln-
Gln-Phe-Phe-Ile-Asp-Phe-Arg-Leu-Ile-Gly-Trp-Ser-Asp-Trp-
Ile-Ile-Ala-Pro-Thr-Gly-Tyr-Tyr-Gly-Asn-Tyr-Cys-Glu-Gly-
Ser-Cys-Pro-Ala-Tyr-Leu-Ala-Gly-Val-Pro-Gly-Ser-Ala-Ser-
Ser-Phe-His-Thr-Ala-Val-Val-Asn-Gln-Tyr-Arg-Met-Arg-Gly-
20   Leu-Asn-Pro-Gly-Thr-Val-Asn-Ser-Cys-Cys-Ile-Pro-Thr-Lys-
Leu-Ser-Thr-Met-Ser-Met-Leu-Tyr-Phe-Asp-Asp-Glu-Tyr-Asn-
Ile-Val-Lys-Arg-Asp-Val-Pro-Asn-Met-Ile-Val-Glu-Glu-Cys-
Gly-Cys-Ala-OH

11. A protein according to Claim 10 wherein
25   said first chain has the formula: H-Ser-Thr-Ala-Pro-Leu-
Pro-Trp-Pro-Trp-Ser-Pro-Ala-Ala-Leu-Arg-Leu-Leu-Gln-Arg-
Pro-Pro-Glu-Glu-Pro-Ala-Val-His-Ala-Asp-Cys-His-Arg-Ala-
Ser-Leu-Asn-Ile-Ser-Phe-Gln-Glu-Leu-Gly-Trp-Asp-Arg-Trp-
Ile-Val-His-Pro-Pro-Ser-Phe-Ile-Phe-His-Tyr-Cys-His-Gly-
30   Gly-Cys-Gly-Leu-Pro-Thr-Leu-Pro-Asn-Leu-Pro-Leu-Ser-Val-
Pro-Gly-Ala-Pro-Pro-Thr-Pro-Val-Gln-Pro-Leu-Leu-Leu-Val-
Pro-Gly-Ala-Gln-Pro-Cys-Cys-Ala-Ala-Leu-Pro-Gly-Thr-Met-
Arg-Ser-Leu-Arg-Val-Arg-Thr-Thr-Ser-Asp-Gly-Gly-Tyr-Ser-
Phe-Lys-Tyr-Glu-Thr-Val-Pro-Asn-Leu-Leu-Thr-Gln-His-Cys-
35   Ala-Cys-Ile-OH.

12. A method of obtaining substantially homogeneous proteins having inhibin activity from crude extracts comprising

procuring extract material having inhibin
5   activity,

exposing said extract to a support medium to which heparin moieties are attached under such conditions that inhibin proteins adsorb to said heparin moieties, and subsequently eluting said inhibin proteins
10   from said heparin moieties,

gel filtrating said eluted inhibin proteins and selecting fractions having inhibin activity, and

fractionating said gel filtrated fractions on at least one reverse-phase high performance liquid
15   chromatography column.

13. A method according to Claim 12 wherein said gel filtrated fractions are fractionated on at least two successive reverse-phase high performance liquid chromatography columns of different solid and/or
20   liquid phase conditions.

14. A method according to Claim 12 wherein at least one of said RP-HPLCS is performed on a C3-C5 column.

15. A method according to Claim 12 wherein
25   said crude extract is obtained from porcine body material.

16. A method according to Claim 15 wherein said body material is porcine follicular fluid.

17. A synthetic protein produced by recombinant DNA techniques or the like having two chains interconnected by disulfide bonding, said first chain having formula: H-Ser-Thr-Ala-Pro-Leu-Pro-Trp-Pro-Trp-Ser-Pro-Ala-Ala-Leu-Arg-Leu-Leu-Gln-Arg-Pro-Pro-Glu-Glu-Pro-Ala-Val-His-Ala-Asp-Cys-His-Arg-Ala-Ser-Leu-Asn-Ile-Ser-Phe-Gln-Glu-Leu-Gly-Trp-Asp-Arg-Trp-Ile-Val-His-Pro-Pro-Ser-Phe-Ile-Phe-His-Tyr-Cys-His-Gly-Gly-Cys-Gly-Leu-Pro-Thr-Leu-Pro-Asn-Leu-Pro-Leu-Ser-Val-Pro-Gly-Ala-Pro-Pro-Thr-Pro-Val-Gln-Pro-Leu-Leu-Leu-Val-Pro-Gly-Ala-Gln-Pro-Cys-Cys-Ala-Ala-Leu-Pro-Gly-Thr-Met-Arg-Ser-Leu-Arg-Val-Arg-Thr-Thr-Ser-Asp-Gly-Gly-Tyr-Ser-Phe-Lys-Tyr-Glu-Thr-Val-Pro-Asn-Leu-Leu-Thr-Gln-His-Cys-Ala-Cys-Ile-OH, and said second chain having either the formula: H-Gly-Leu-Glu-Cys-Asp-Gly-Lys-Val-Asn-Ile-Cys-Cys-Lys-Lys-Gln-Phe-Phe-Val-Ser-Phe-Lys-Asp-Ile-Gly-Trp-Asn-Asp-Trp-Ile-Ile-Ala-Pro-Ser-Gly-Tyr-His-Ala-Asn-Tyr-Cys-Glu-Gly-Glu-Cys-Pro-Ser-His-Ile-Ala-Gly-Thr-Ser-Gly-Ser-Ser-Leu-Ser-Phe-His-Ser-Thr-Val-Ile-Asn-His-Tyr-Arg-Met-Arg-Gly-His-Ser-Pro-Phe-Ala-Asn-Leu-Lys-Ser-Cys-Cys-Val-Pro-Thr-Lys-Leu-Arg-Pro-Met-Ser-Met-Leu-Tyr-Tyr-Asp-Asp-Gly-Gln-Asn-Ile-Ile-Lys-Lys-Asp-Ile-Gln-Asn-Met-Ile-Val-Glu-Glu-Cys-Gly-Cys-Ser-OH, or the formula H-Gly-Leu-Glu-Cys-Asp-Gly-Arg-Thr-Asn-Leu-Cys-Cys-Arg-Gln-Gln-Phe-Phe-Ile-Asp-Phe-Arg-Leu-Ile-Gly-Trp-Ser-Asp-Trp-Ile-Ile-Ala-Pro-Thr-Gly-Tyr-Tyr-Gly-Asn-Tyr-Cys-Glu-Gly-Ser-Cys-Pro-Ala-Tyr-Leu-Ala-Gly-Val-Pro-Gly-Ser-Ala-Ser-Ser-Phe-His-Thr-Ala-Val-Val-Asn-Gln-Tyr-Arg-Met-Arg-Gly-Leu-Asn-Pro-Gly-Thr-Val-Asn-Ser-Cys-Cys-Ile-Pro-Thr-Lys-Leu-Ser-Thr-Met-Ser-Met-Leu-Tyr-Phe-Asp-Asp-Glu-Tyr-Asn-Ile-Val-Lys-Arg-Asp-Val-Pro-Asn-Met-Ile-Val-Glu-Glu-Cys-Gly-Cys-Ala-OH

18. A synthetic polypeptide useful as a
chemical intermediate for the production of a bio-
logically active dimer, which polypeptide is selected
from the group consisting of: (a) H-Ser-Thr-Ala-Pro-
5   Leu-Pro-Trp-Pro-Trp-Ser-Pro-Ala-Ala-Leu-Arg-Leu-Leu-Gln-
Arg-Pro-Pro-Glu-Glu-Pro-Ala-Val-His-Ala-Asp-Cys-His-Arg-
Ala-Ser-Leu-Asn-Ile-Ser-Phe-Gln-Glu-Leu-Gly-Trp-Asp-Arg-
Trp-Ile-Val-His-Pro-Pro-Ser-Phe-Ile-Phe-His-Tyr-Cys-His-
Gly-Gly-Cys-Gly-Leu-Pro-Thr-Leu-Pro-Asn-Leu-Pro-Leu-Ser-
10   Val-Pro-Gly-Ala-Pro-Pro-Thr-Pro-Val-Gln-Pro-Leu-Leu-Leu-
Val-Pro-Gly-Ala-Gln-Pro-Cys-Cys-Ala-Ala-Leu-Pro-Gly-Thr-
Met-Arg-Ser-Leu-Arg-Val-Arg-Thr-Thr-Ser-Asp-Gly-Gly-Tyr-
Ser-Phe-Lys-Tyr-Glu-Thr-Val-Pro-Asn-Leu-Leu-Thr-Gln-His-
Cys-Ala-Cys-Ile-OH; (b) H-Gly-Leu-Glu-Cys-Asp-Gly-Arg-
15   Thr-Asn-Leu-Cys-Cys-Arg-Gln-Gln-Phe-Phe-Ile-Asp-Phe-Arg-
Leu-Ile-Gly-Trp-Ser-Asp-Trp-Ile-Ile-Ala-Pro-Thr-Gly-Tyr-
Tyr-Gly-Asn-Tyr-Cys-Glu-Gly-Ser-Cys-Pro-Ala-Tyr-Leu-Ala-
Gly-Val-Pro-Gly-Ser-Ala-Ser-Ser-Phe-His-Thr-Ala-Val-Val-
Asn-Gln-Tyr-Arg-Met-Arg-Gly-Leu-Asn-Pro-Gly-Thr-Val-Asn-
20   Ser-Cys-Cys-Ile-Pro-Thr-Lys-Leu-Ser-Thr-Met-Ser-Met-Leu-
Tyr-Phe-Asp-Asp-Glu-Tyr-Asn-Ile-Val-Lys-Arg-Asp-Val-Pro-
Asn-Met-Ile-Val-Glu-Glu-Cys-Gly-Cys-Ala-OH and (c) H-Gly-
Leu-Glu-Cys-Asp-Gly-Lys-Val-Asn-Ile-Cys-Cys-Lys-Lys-Gln-
Phe-Phe-Val-Ser-Phe-Lys-Asp-Ile-Gly-Trp-Asn-Asp-Trp-Ile-
25   Ile-Ala-Pro-Ser-Gly-Tyr-His-Ala-Asn-Tyr-Cys-Glu-Gly-Glu-
Cys-Pro-Ser-His-Ile-Ala-Gly-Thr-Ser-Gly-Ser-Ser-Leu-Ser-
Phe-His-Ser-Thr-Val-Ile-Asn-His-Tyr-Arg-Met-Arg-Gly-His-
Ser-Pro-Phe-Ala-Asn-Leu-Lys-Ser-Cys-Cys-Val-Pro-Thr-Lys-
Leu-Arg-Pro-Met-Ser-Met-Leu-Tyr-Tyr-Asp-Asp-Gly-Gln-Asn-
30   Ile-Ile-Lys-Lys-Asp-Ile-Gln-Asn-Met-Ile-Val-Glu-Glu-Cys-
Gly-Cys-Ser-OH.

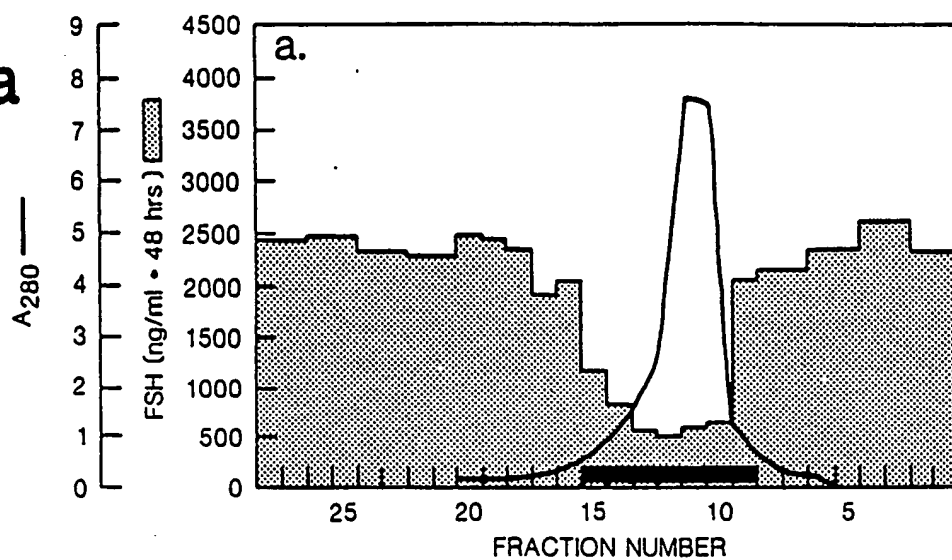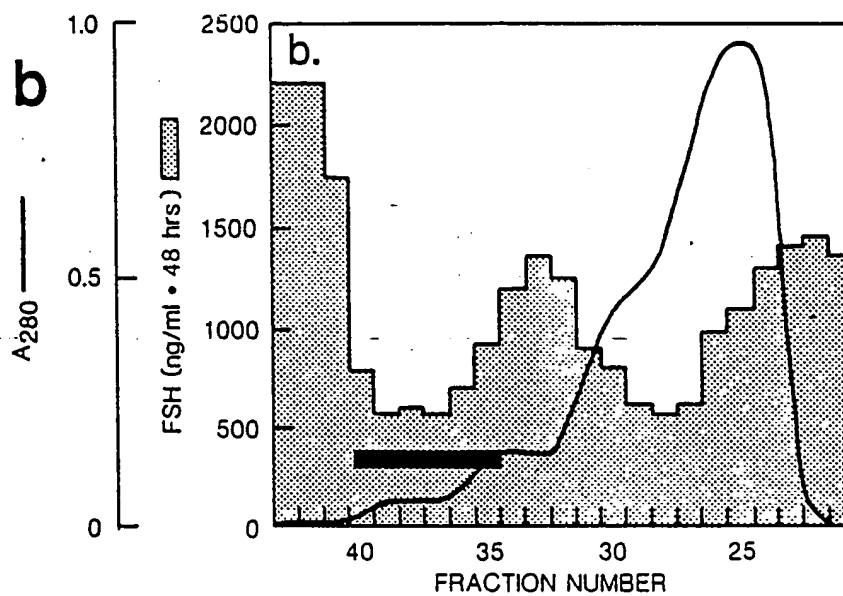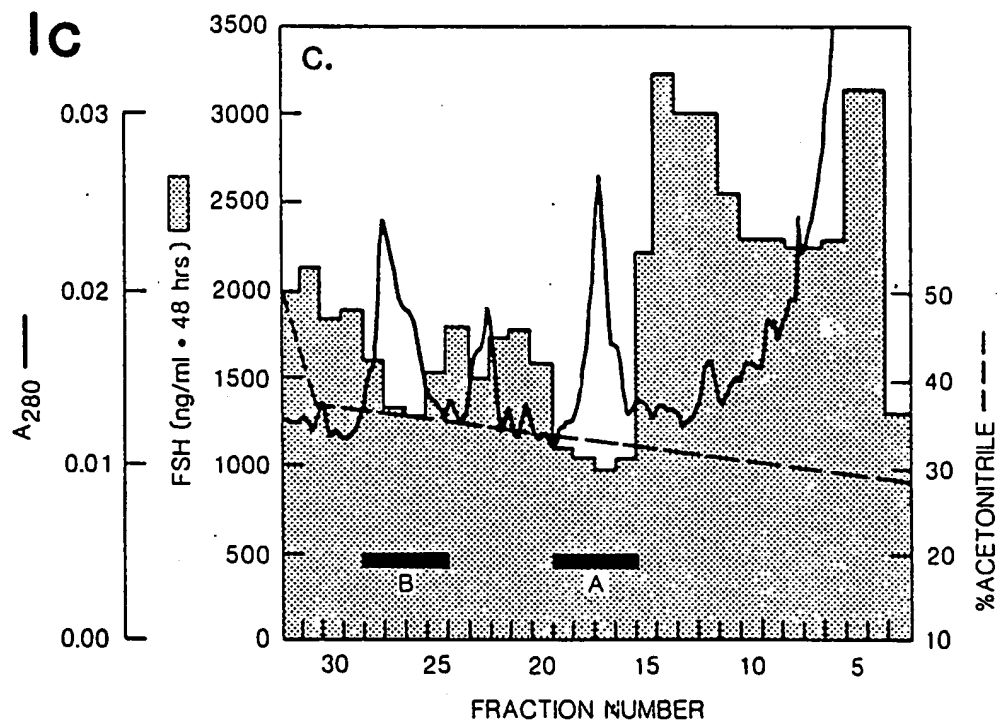19. A polypeptide according to Claim 18 having
the formula: H-Gly-Leu-Glu-Cys-Asp-Gly-Arg-Thr-Asn-Leu-
Cys-Cys-Arg-Gln-Gln-Phe-Phe-Ile-Asp-Phe-Arg-Leu-Ile-Gly-
Trp-Ser-Asp-Trp-Ile-Ile-Ala-Pro-Thr-Gly-Tyr-Tyr-Gly-Asn-
5   Tyr-Cys-Glu-Gly-Ser-Cys-Pro-Ala-Tyr-Leu-Ala-Gly-Val-Pro-
Gly-Ser-Ala-Ser-Ser-Phe-His-Thr-Ala-Val-Val-Asn-Gln-Tyr-
Arg-Met-Arg-Gly-Leu-Asn-Pro-Gly-Thr-Val-Asn-Ser-Cys-Cys-
Ile-Pro-Thr-Lys-Leu-Ser-Thr-Met-Ser-Met-Leu-Tyr-Phe-Asp-
Asp-Glu-Tyr-Asn-Ile-Val-Lys-Arg-Asp-Val-Pro-Asn-Met-Ile-
10 Val-Glu-Glu-Cys-Gly-Cys-Ala-OH.

20. A polypeptide according to Claim 18 having
the formula: H-Gly-Leu-Glu-Cys-Asp-Gly-Lys-Val-Asn-Ile-
Cys-Cys-Lys-Lys-Gln-Phe-Phe-Val-Ser-Phe-Lys-Asp-Ile-Gly-
Trp-Asn-Asp-Trp-Ile-Ile-Ala-Pro-Ser-Gly-Tyr-His-Ala-Asn-
15 Tyr-Cys-Glu-Gly-Glu-Cys-Pro-Ser-His-Ile-Ala-Gly-Thr-Ser-
Gly-Ser-Ser-Leu-Ser-Phe-His-Ser-Thr-Val-Ile-Asn-His-Tyr-
Arg-Met-Arg-Gly-His-Ser-Pro-Phe-Ala-Asn-Leu-Lys-Ser-Cys-
Cys-Val-Pro-Thr-Lys-Leu-Arg-Pro-Met-Ser-Met-Leu-Tyr-Tyr-
Asp-Asp-Gly-Gln-Asn-Ile-Ile-Lys-Lys-Asp-Ile-Gln-Asn-Met-
20 Ile-Val-Glu-Glu-Cys-Gly-Cys-Ser-OH.

FIG. 1a

FIG. 1b

FIG. 1c

FIG. 2a

FIG. 2b

FIG. 2c
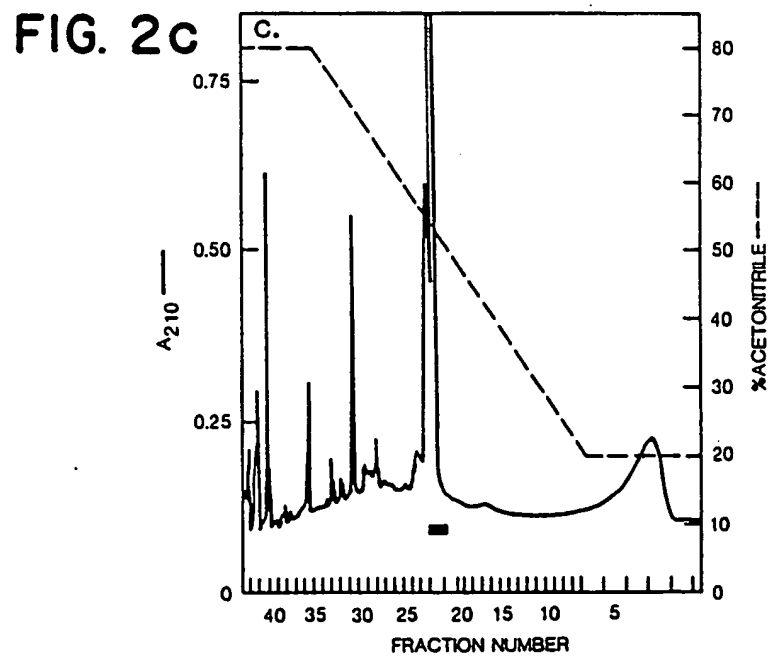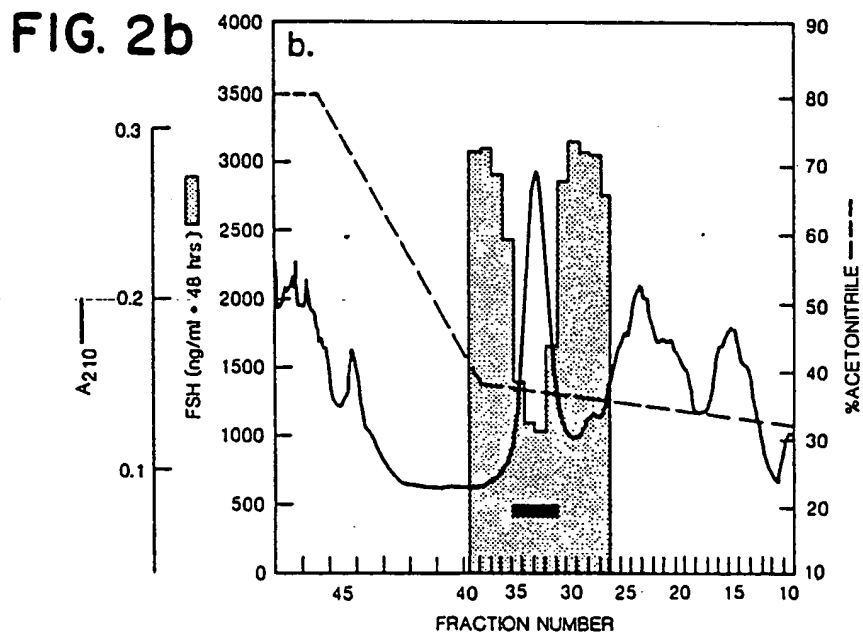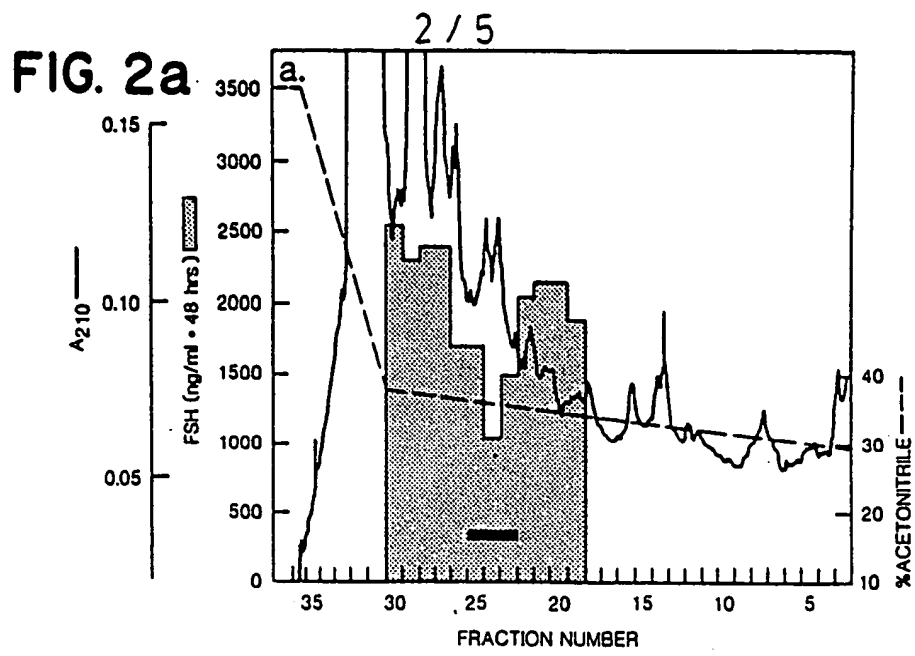
FIG. 3
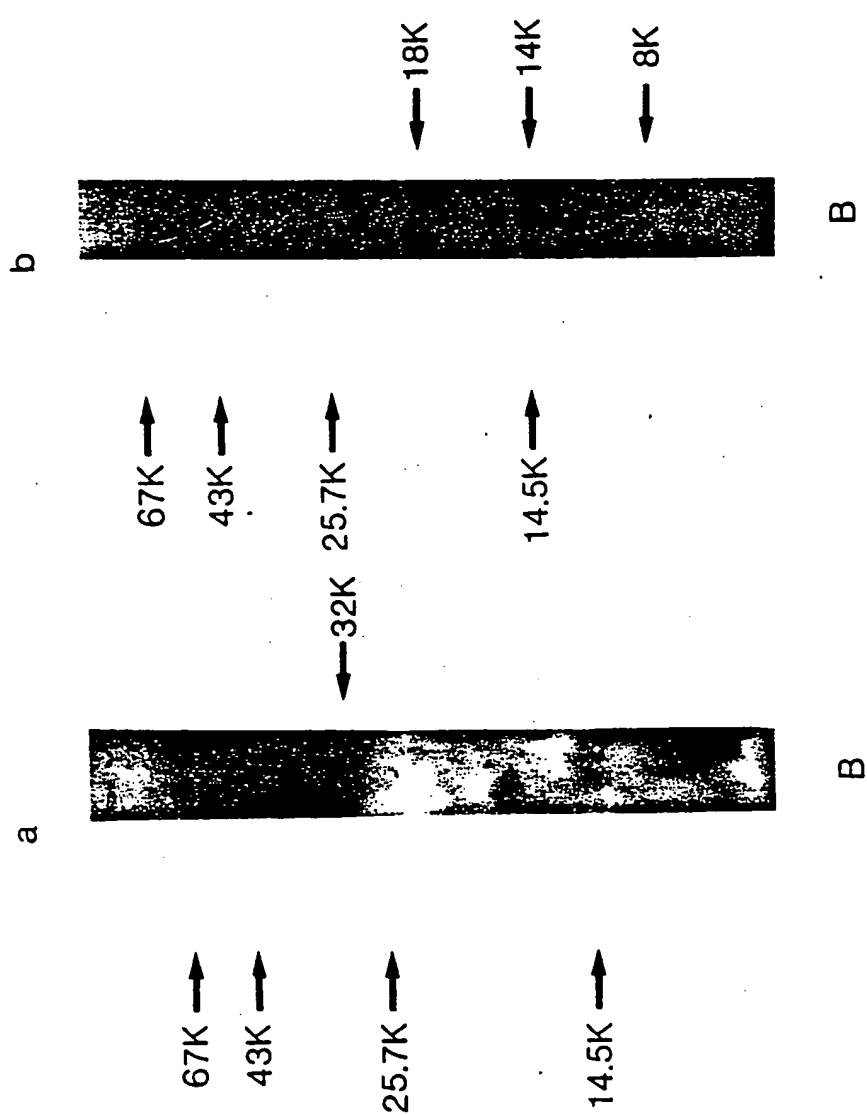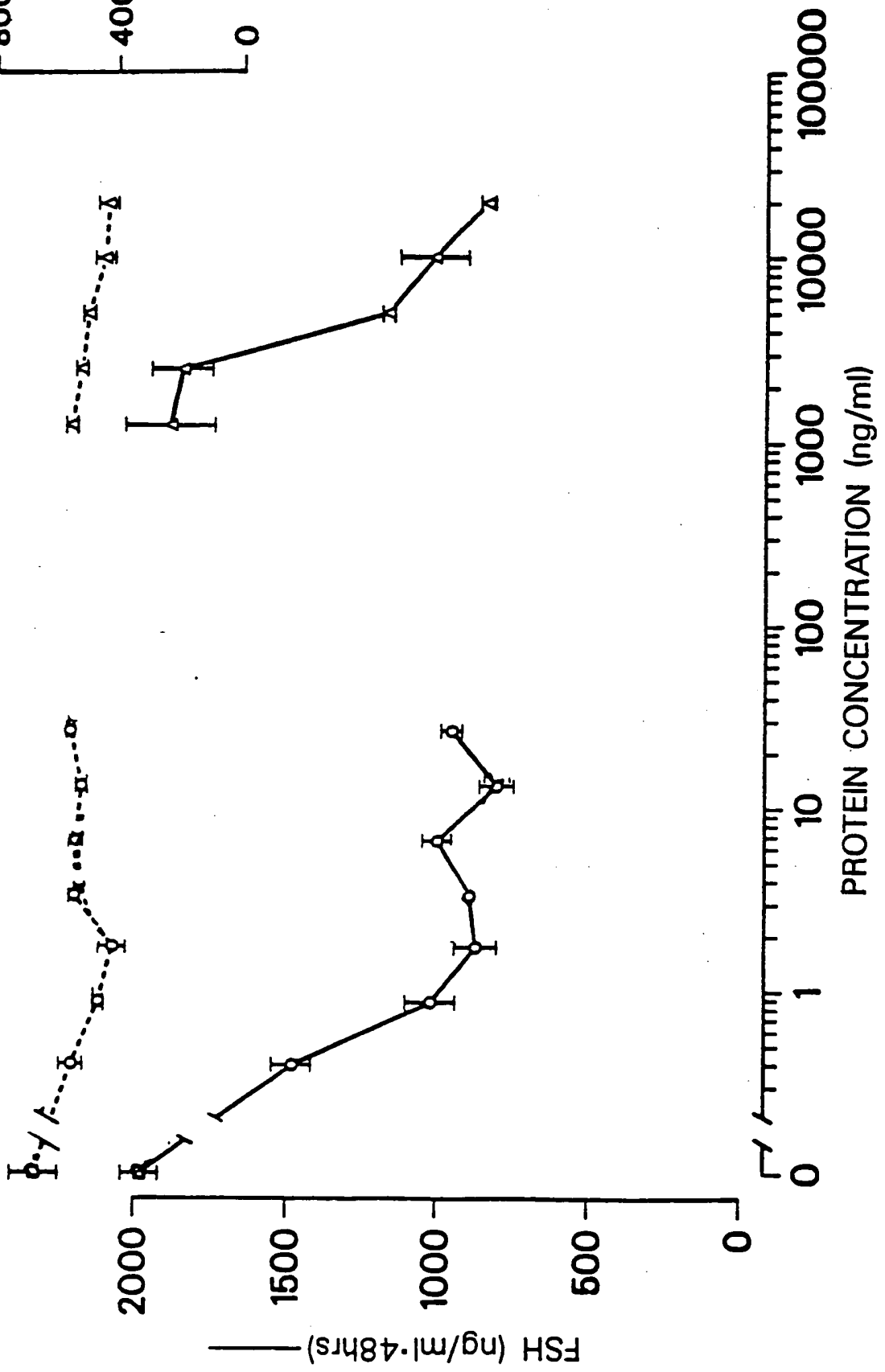
FIG. 4

FIG. 5a
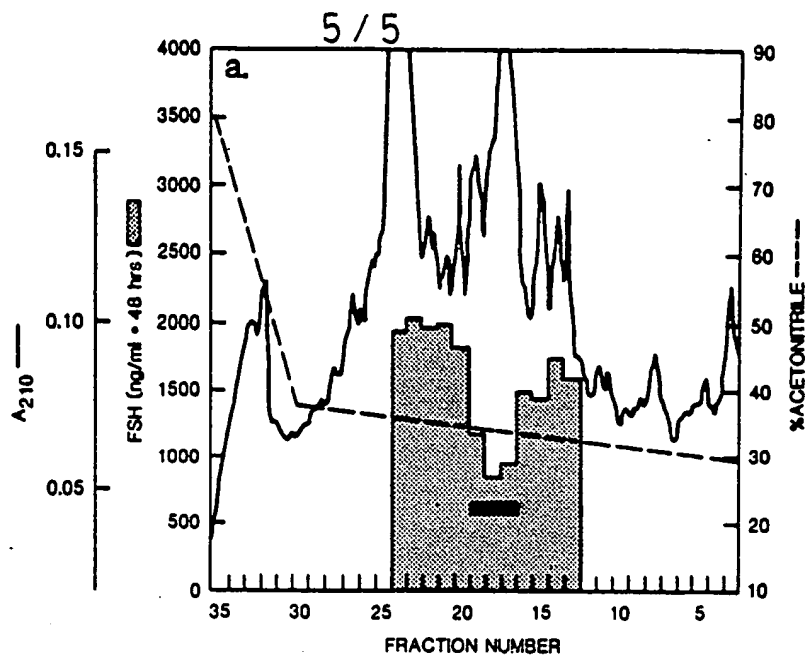
FIG. 5b

FIG. 5c

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 3

According to International Patent Classification (IPC) or to both National Classification and IPC

INT CL.4 C07K 7/06, 7/08, 7/10, 13/00, 15/00
US CL. 530/313, 324, 325, 326, 327, 328, 344

II. FIELDS SEARCHED

Minimum Documentation Searched 4

Classification System	Classification Symbols
U.S.	530/313, 324, 325, 326, 327, 328, 344

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 5

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
X	US,N, Biochemical & Biophysical Research Communication, Published 14 JUNE 1985, Vol. 129, No.2, MIYAMOTO et al "Isolation of Porcine Follicular Fluid Inhibin of 32K Daltons," pages 396-403.	1-20
A	US,N, Archives of Andrology, Published 13 DECEMBER 1984, Vol.13, CHANNING et al "Porcine Inhibin: Initial Fractionation as a High Molecular Weight Complex," p.219-231.	1,12-16
A	US,N, Journal of Chromatography, Published 1984, Vol.288, RIVIER et al "Reversed-Phase High-Performance Liquid Chromatography Preparative Purification of Synthetic Peptides," pp. 303-328.	1,12-16
A	US,N, Biology of Reproduction, Published 1986, Vol.34, MILLS et al "The Effects of Porcine Follicular Fluid Inhibin on Gonadotropin Secretion in the Rabbit," pp. 336-343.	1

* Special categories of cited documents: 15

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 2	Date of Mailing of this International Search Report 2
12 AUGUST 1986	29 AUG 1986
International Searching Authority 1	Signature of Authorized Officer 20
ISA/US	NATHAN M. NUTTER

III. DOCUMENTS CONSIDERED TO BE RELEVANT	(CONTINUED FROM THE SECOND SHEET)	
Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No 18
A	US,P, Chem Abst. 103: 65074s, Published 02 SEPTEMBER 1985, Vol. 103, No.9 "Isolation From Porcine Follicular Fluid of a Potent Inhibin-like Biological Activity," RIVIER et al.	1-20